Nicotinic Receptor Function in the Mammalian Central Nervous System[a]

EDSON X. ALBUQUERQUE,[b,c] EDNA F. R. PEREIRA,[b,c]
NEWTON G. CASTRO,[b,c] MANICKAVASAGOM ALKONDON,[b]
SIGRID REINHARDT,[d] HANNSJÖRG SCHRÖDER,[e]
AND ALFRED MAELICKE[d]

[b]*Department of Pharmacology and Experimental Therapeutics*
University of Maryland School of Medicine
Baltimore, Maryland 21201

[c]*Laboratory of Molecular Pharmacology II*
Institute of Biophysics "Carlos Chagas Filho"
Federal University of Rio de Janeiro
Rio de Janeiro, RJ 21944, Brazil

[d]*Institute of Physiological Chemistry and Pathobiochemistry*
Johannes-Gutenberg University Medical School
Duesbergweg 6
Mainz, Germany

[e]*Department of Anatomy*
University of Köln
Köln, Germany

Earlier studies on the characterization of nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS) relied on assays of the binding of nicotinic radioligands to various brain regions by the use of radioligand binding assays.[1–5] However, it was not until very recently that the functional properties of some of these receptors could be electrophysiologically addressed.[6–20]

Initially, on the basis of the binding of [^{3}H]nicotine and [^{125}I]α-bungarotoxin (α-BGT) to various brain regions, it was demonstrated that two distinct populations of presumed nAChRs existed in the CNS.[2,3] Neuronal nAChRs that could bind [^{3}H]nicotine with high affinity were identified in the interpeduncular nucleus, superior colliculus, medial habenula, substantia nigra pars compacta and ventral tegmental area, molecular layer of the dentate gyrus, presubiculum, cerebral cortex, and most of the thalamic nuclei.[3] Studies carried out in brain synaptosomes led to the suggestion that these neuronal nAChRs were localized mostly presynaptically and were responsible for the control of transmitter release in various neurotransmitter

[a]This work was supported in the United States by National Institutes of Health grants NS25296 and ES05730, National Institute of Mental Health Center for Neuroscience and Schizophrenia grant P-50MH44211, and a Conselho Nacional de Pesquisa e Desenvolvimento graduate student fellowship from Brazil. In Germany, this work was supported by DFG grant Ma 599/17-1 and a grant from Fonds der Chemischen Industrie.
[b]Address to which reprint requests should be sent.

systems.[21–23] On the other hand, [^{125}I]α-BGT was found to label several brain areas, namely, the cerebral cortex, hippocampus, hypothalamus, inferior and superior colliculus, and some brain-stem nuclei,[3–5] whereas no α-BGT-sensitive nicotinic responses could be found in these areas.[24] For this reason, α-BGT-binding proteins at one time were believed to mediate functions unrelated to nAChR.[25] Further, it was not completely clear whether nAChRs binding [^{3}H]nicotine with high affinity consisted of a homogenous population of a single nAChR subtype, or a population of various subtypes of nAChRs that in spite of being distinct from one another could have similar affinities for [^{3}H]nicotine. Several complementary experimental approaches, including ligand-binding assays, molecular biological procedures, and electrophysiological techniques were necessary to unveil the functional, pharmacological, and structural diversity of CNS nAChRs.[26,27]

Like their counterparts in the muscle, the nAChRs in the CNS are ligand-gated cation channels. Inasmuch as neuronal and muscle nAChRs are encoded by homologous genes, cDNAs encoding muscle nAChR subunits have been used to screen libraries of mRNAs isolated from neurons from various CNS areas of chick, rat, and humans. To date, at least eight α- (α2 through α9) and three β- (β2 through β4) subunits have been cloned from chick, rat, and human neuronal tissues.[26,28] A CNS nAChR subunit is classified as α if it has the characteristic ACh-binding domain found in the muscle nAChR α-subunit, that is, the vicinal Cys residues in the N-terminal region. The CNS nAChR β-subunits comprise a group of proteins that, in addition to playing an important role in defining the structure of the receptor, may also contribute to the sensitivity of the nAChR to a number of pharmacological agents. Whereas the CNS nAChR α-subunits are considerably homologous with their muscle counterparts, the CNS nAChR β-subunits show very low homology to the muscle nAChR β-subunit.[29] It is most likely that the diverse properties of CNS nAChRs are largely due to the fact that these receptors can be made up of different associations of α- and β-subunits. Indeed, transient expression of a variety of combinations of α- and β-subunits can give rise to a number of functionally distinct neuronal nAChRs in *Xenopus* oocytes.[30] Molecular biological studies have also proven that α7, α8, and α9 nAChR subunits can form homomeric, functional nAChR channels that are remarkably and uniquely sensitive to blockade by α-BGT.[28,31,32]

Although several studies have dealt with the characterization of recombinant neuronal nAChRs,[26] it was not until recently that studies were directed at characterizing functionally and pharmacologically native CNS nAChRs *in situ*.[27] Research in our laboratory has been aimed at identifying the various functional nAChRs expressed throughout the brain, at understanding the mechanisms by which nAChR activity can be modulated, and at defining the ion selectivity of α-BGT-sensitive CNS nAChRs. In the present paper, the following topics are discussed: (1) how CNS nAChR subtypes can be identified on the basis of the pharmacological and kinetic properties of nicotinic currents activated in various CNS neuronal preparations, (2) the modulation of nAChR activity via an ACh-insensitive binding site and via intracellular mechanisms, and (3) the ion permeability of α-BGT-sensitive CNS nAChRs. An understanding of the pharmacological and functional characteristics of CNS nAChRs may serve as the cornerstone for the development of useful therapeutic strategies to treat and/or prevent neuropathological conditions such as Alzheimer's[33] and Parkinson's[34] diseases and nicotine addiction, in which the function of CNS nicotinic systems is known to be severely compromised, and will help to unveil the physiological roles of nicotinic synaptic transmission in the brain.

CHARACTERIZATION OF FUNCTIONAL nAChRs IN MAMMALIAN CNS NEURONS

By the end of the 1980s, molecular biological studies revealed that mRNAs coding for several nAChR α- and β-subunits can be found in various regions of the CNS,[26] and it was not until the early 1990s that reports regarding the identification and characterization of functional nAChRs in the mammalian CNS by electrophysiological techniques started to appear.[8–10,12,13] Previously, only two studies had reported the existence of functional nAChRs in CNS neurons by direct electrophysiological techniques.[6,7] Our studies on the characterization of hippocampal nAChRs began in 1987 when we demonstrated, for the first time, that nicotinic agonists could activate single-channel currents in cultured hippocampal neurons patch-clamped under the cell-attached condition.[6] The relative difficulty in recording nicotinic currents from CNS neuronal preparations may have contributed to there being until recently practically no electrophysiological studies of nicotinic responses in CNS neurons. Some of the difficulties underlying studies of neuronal nicotinic currents were overcome by the development of devices that could guarantee that nicotinic agonists could be rapidly applied to and immediately removed from the vicinity of the cells.[12,13] The device we initially used consisted of a U-shaped tube fashioned from a thin capillary glass. At the apex of the U tube a pore of 250–400 μm in diameter was made, and through this pore the test solutions were delivered to and removed from the vicinity of the neurons.[13,17] Using this device and others similar to it, it was possible to measure reliably nicotinic responses in biological preparations of CNS neurons.[12–19]

In our initial studies on nicotinic responses in CNS neurons, we found that α-cobratoxin[10] or α-BGT[13] could block nicotinic whole-cell currents activated in cultured hippocampal neurons, demonstrating unequivocally for the first time the existence of α-BGT-sensitive nicotinic currents in CNS neurons. In these neurons, which were cultured from the hippocampi of rat fetuses (embryonic day 17–18), the peak-current amplitude increased with the number of days the neurons were maintained in culture and leveled off by 4–5 weeks after the plating of the neurons.[13] For instance, if a neuron was considered to display nicotinic sensitivity when it responded to anatoxin-a (AnTX, 10 μM) with a peak whole-cell current of at least 10 pA at −50 mV, about 33% of the 6–14-day-old cultured neurons, 88% of the 21–22-day-old cultured neurons, and 98% of the 25–50-day-old cultured neurons showed nicotinic sensitivity.[13] When the hippocampi were removed from the brain of postnatal rats at various ages and the neurons were acutely dissociated by mechanical means, a similar pattern of increase in the peak amplitude of the nicotinic currents was observed.[14]

The pharmacological identification of functional hippocampal nAChRs sensitive to α-BGT relied on the α-BGT-induced blockade of whole-cell currents activated by nicotinic agonists. However, the slowness of onset and pseudoirreversible nature of the blockade induced by this toxin limited its utility. Under the experimental conditions used, the peak amplitude of α-BGT-sensitive nicotinic currents had a tendency to run down with time. This rundown, whose magnitude was variable from cell to cell, was apparently independent of agonist identity and concentration.[13,15] It was then questioned whether the substantial decrease of the peak amplitude of hippocampal nicotinic currents observed after incubation of the neurons with α-BGT reflected the effect of the toxin or rundown of the nicotinic responses. Therefore, we had to search for a new antagonist that could specifically and reversibly block α-BGT-sensitive CNS nAChRs.

Methyllycaconitine (MLA), an alkaloid isolated from the seeds of *Delphinium brownii,* was initially shown to inhibit specifically and potently the binding of [^{125}I]α-BGT to brain membrane preparations.[35] Only at very high concentrations could MLA antagonize the binding of [^{3}H]nicotine to the brain membrane preparations or the binding of α-BGT to muscle nAChRs.[35] Although MLA could reliably be used to identify α-BGT-sensitive CNS nAChRs in binding studies, its mechanisms of action on CNS nAChRs remained unknown until its effects on nicotinic currents activated in cultured hippocampal neurons were investigated. When applied to the neurons via the bath perfusion, MLA could specifically and reversibly decrease the peak amplitude of whole-cell currents elicited by nicotinic agonists.[15] The onset of the MLA effect was very rapid. At concentrations of MLA that could completely inhibit nicotinic currents, the responses of the neurons to *N*-methyl-D-aspartate (NMDA), quisqualate, kainate, or GABA remained unchanged. The IC_{50} for MLA in inhibiting nicotinic currents in hippocampal neurons was found to be about 150

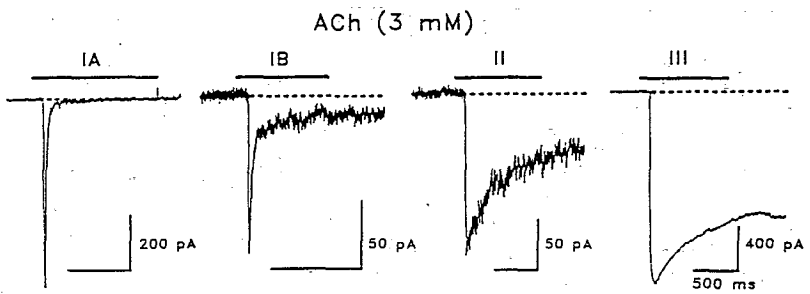

FIGURE 1. Family of ACh-activated whole-cell currents in cultured hippocampal neurons. Sample recordings of whole-cell currents evoked in four hippocampal neurons cultured for 20 days. Recordings were made 4–18 min after obtaining the whole-cell patch. Holding potential = −56 mV. ACh (3 mM) pulses were applied for 0.5 to 1 s as indicated by the solid bars on the top of the traces. The external bath solution (pH, 7.3; osmolarity, 330 mosm) consisted of NaCl 165 mM, KCl 5 mM, $CaCl_2$ 2 mM, glucose 10 mM, 4-(2-hydroethyl)-1-piperazineethanesulfonic acid (HEPES) 5 mM, and tetrodotoxin (TTX) 0.3 μM. The internal pipette solution (pH, 7.3; osmolarity, 340 mosm) consisted of CsCl 80 mM, CsF 80 mM, ethyleneglycoltetraacetic acid (EGTA) 10 mM, and HEPES 10 mM.

pM, and the inhibition caused by MLA was competitive with that caused by α-BGT. Therefore, MLA is a potent, reversible, and competitive nicotinic antagonist specific for α-BGT-sensitive CNS nAChRs.[15,17]

The knowledge that accumulated throughout the years on the characteristics of recombinant and native CNS nAChRs prompted us to address in detail the diversity of CNS nAChRs expressed in various brain regions.[17,19,36] Using a number of nicotinic agonists and antagonists, we were able to demonstrate that at least three distinct types of nicotinic currents can be elicited in hippocampal neurons. According to their pharmacological and kinetic properties, these currents were classified into types IA, II, and III[17,19] (see FIG. 1). It was suggested that each of these currents is carried by a distinct type of CNS nAChR channel.

Type IA currents, which are the predominant nicotinic responses that can be elicited in hippocampal neurons, desensitize fast, have low affinity for ACh, and are highly sensitive to blockade by the neurotoxins α-BGT and MLA.[17] Based on EC_{50}s

in eliciting type IA currents, AnTX was the most potent agonist, followed by dimethylphenylpiperazinium (DMPP), (−)nicotine, cytisine, ACh, carbachol, and (+)nicotine. At saturating concentrations of any given nicotinic agonist tested, the decay phase of type IA currents at a holding potential of −50 mV could be fit by a double-exponential function, with the fast decay-time constant being as short as 6 ms and averaging 26.7 ± 1.9 ms. In outside-out patches excised from the soma of hippocampal neurons that showed at least 50 pA of acetylcholine (ACh, 1 mM)-elicited type IA currents, ACh activated a type of single channel whose open time is about 110 μs at −60 mV and that has a conductance of 73 pS.[18] This type of ACh-activated channel is sensitive to blockade by MLA, and is consistently found in outside-out patches excised from neurons that display type IA whole-cell currents. In addition, these single-channel currents show the fast kinetics of activation and inactivation characteristic of type IA currents. Thus, single channels that inactivate rather fast, have a high conductance, and a brief lifetime account for the α-BGT-sensitive IA currents.[18] Because the activation and desensitization rates of these channels are so similar, the response is always transient, and its magnitude depends upon the rate of agonist application. This may explain the failure of previous studies, which used slow agonist perfusion, to detect the brief-lifetime, fast-inactivating, α-BGT-sensitive single-channel currents.

Only about 10% of the cultured hippocampal neurons studied to date have exhibited type II and III nicotinic currents.[16,18] These currents desensitize relatively slowly, are relatively insensitive to blockade by α-BGT or MLA, and can be selectively blocked by either dihydro-β-erythroidine (DHβE, type II currents) or mecamylamine (type III currents). A distinct rank order of potency of agonists was found for each of these two types of nicotinic currents. Whereas the most potent agonist in eliciting type II currents was ACh, followed by AnTX, (−) nicotine, DMPP, carbamylcholine, cytisine, and (+) nicotine, the most potent agonist in eliciting type III currents was AnTX, followed by cytisine, (−)nicotine, DMPP, ACh, carbamylcholine, and (+)nicotine.[17] In some neurons, whole-cell currents with characteristics of both type IA and type II currents have been observed (FIG. 1). These composite currents, which are referred to as IB, display a rapidly decaying component that can be specifically blocked by α-BGT or MLA, and a slowly decaying component that can be specifically blocked by DHβE.[17] In addition, fast application of ACh (1 mM) to a few outside-out patches excised from hippocampal neurons can activate simultaneously two distinct types of single channels: a fast-desensitizing, brief-lifetime, and high-conductance channel that accounts for the type IA current, and a slowly desensitizing, long-lifetime, and low-conductance channel that accounts for the type II currents.[18] Although these findings indicate that some neurons can express two types of nAChRs and that in some cases both nAChR subtypes can be expressed in the same region of the neuron, it is still unclear whether topological segregation of the two nAChR subtypes in the neuronal soma, dendrites, or axon also takes place.

The kinetic and pharmacological properties of the three "pure" types of whole-cell currents activated by nicotinic agonists in hippocampal neurons led us to suggest that structurally distinct nAChRs subserved each of these currents.[16] Comparison of the properties of currents evoked by activation of recombinant nAChRs expressed in oocytes with those of the nicotinic whole-cell currents activated in hippocampal neurons led us to hypothesize that an α7-bearing nAChR accounted for the type IA response, an α4β2 nAChR for the type II response, and an α3β4 nAChR for the type III response.[17] These inferences were supported by the results obtained in *in situ* hybridization studies using digoxigenin-labeled cDNA probes specific for nAChR α7, α4, and β2 subunits. The mRNAs coding for CNS nAChR α7, α4, and β2 subunits

were found to be expressed in cultured hippocampal neurons[19] (FIG. 2), the α7- and α4-subunit mRNAs being detected mostly in the perinuclear cytosol of the cultured cells. In addition, the α7-subunit mRNA was found to be expressed in about 70–80% of all cells, whereas the α4-subunit mRNA was found to be expressed in only 20–30% of the cells, indicating that the proportion of neurons that express the α7- or α4-subunit mRNAs is strongly correlated with the probability of eliciting type IA or type II currents. Nevertheless, it remains unclear whether type IA currents are subserved by a homomeric α7 nAChR or a heteromeric nAChR that bears in its structure the α7 subunit, because whereas the kinetic and pharmacological properties of IA responses are the same as those of currents evoked by activation of homomeric α7 nAChRs transiently expressed in oocytes, some of the properties of the ion channels that account for IA responses are quite different from those of

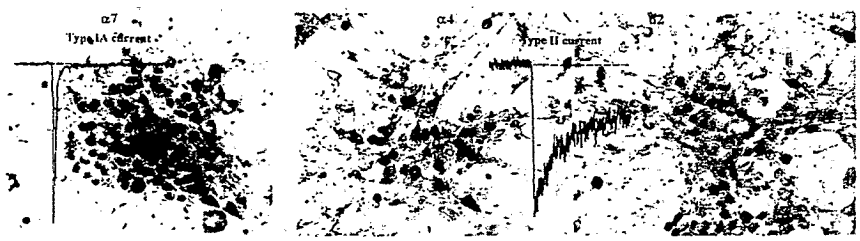

FIGURE 2. Identification of nAChR subunit mRNAs expressed in rat cultured hippocampal neurons. **Left panel:** Expression of α7-subunit mRNA in cultured hippocampal neurons as revealed by *in situ* hybridization using a specific digoxigenin-labeled cDNA probe for 350 bps (1617–1968) of the 3′ end. A sample recording of a type IA current recorded from a hippocampal neuron cultured for 20 days and held at −56 mV is superimposed. As stated in the text, the characteristics of this nicotinic current resembles those of currents triggered by activation of homomeric α7 nAChRs in *Xenopus* oocytes. **Middle and right panels:** Expression of α4-subunit mRNA (*middle*) and β4-subunit mRNA (*right*) in cultured hippocampal neurons as revealed by *in situ* hybridization using specific digoxigenin-labeled cDNA probes for 202 bps (1903–2105) of the 3′ end of the α4-subunit mRNA and for 205 bps (1–205) of the 5′ end of the β2-subunit mRNA. A sample recording of a type II current recorded from a hippocampal neuron cultured for 20 days and held at −56 mV is superimposed to the middle and right panels. As stated in the text, the characteristics of this nicotinic current resembles those of currents triggered by activation of α4β2 nAChRs in various expression systems. Methodology used has been described elsewhere.

homomeric α7-nAChR channels expressed in oocytes.[18,37] For instance, whereas the single channels that account for type IA currents appear as isolated, short-lived events, homomeric α7 nAChRs expressed in oocytes are typically activated in bursts of 10-ms duration.[37] These functional discrepancies indicate that posttranslational modifications and/or actual subunit compositions differ between the native α-BGT-sensitive hippocampal nAChR and the homomeric α7 nAChR expressed in oocytes. It is also conceivable that these discrepancies could be accounted for by the membrane compositions of *Xenopus* oocytes and hippocampal neurons.

We also demonstrated that ACh and other nicotinic agonists can activate fast-desensitizing, MLA-/α-BGT-sensitive whole-cell currents in cultured neurons from the rat olfactory bulb[36] (FIG. 3). The pharmacological and kinetic profile of these nicotinic currents suggested that olfactory bulb neurons express a neuronal

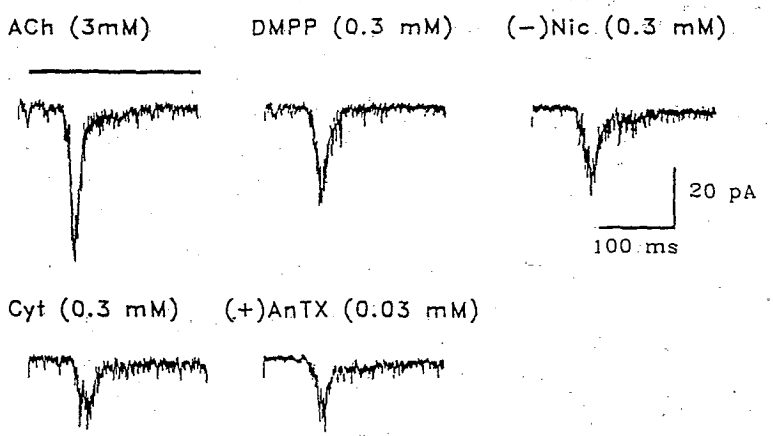

FIGURE 3. Nicotinic currents activated in rat cultured olfactory bulb neurons. Sample recordings of whole-cell currents evoked in one olfactory bulb neuron cultured for 29 days. The nicotinic agonists were applied to the cell as 250-ms pulses separated by 2-min intervals. Holding potential = −56 mV. Compositions of the external and internal solutions were the same as those described in FIGURE 1.

nAChR with properties similar to those reported above for α-BGT-sensitive nAChRs expressed in hippocampal neurons. To date, no other type of nicotinic response has been found in cultured olfactory bulb neurons, although mRNAs encoding for α4 and β2 have been found in these neurons by *in situ* hybridization (unpublished observations).

The discovery of substances that can act specifically on one receptor type in the CNS is of immense therapeutic implications given that such substances would have more selective neurological effects and as a result may be less toxic. Very recently, the alkaloid epibatidine, which was originally isolated from frog skin, was shown to have a potent analgesic activity that is insensitive to naloxone, but can be completely antagonized by the nicotinic antagonist mecamylamine.[38] The idea that CNS nAChRs could mediate the analgesic activity of epibatidine was supported by the finding that this alkaloid can induce mecamylamine-sensitive ion fluxes into cultured cells that express different neuronal nAChR subtypes.[38] The (−) and (+) stereoisomers of epibatidine were approximately equipotent as nicotinic agonists in the tested cell systems.[38] To characterize the specificity of epibatidine on CNS nAChRs, we tested the effects of both isomers in cultured hippocampal neurons under whole-cell patch-clamp conditions. Both (+) and (−) epibatidine were able to act as full agonists in eliciting type IA currents (FIG. 4), and practically no stereoselectivity was observed, because the EC_{50}s for (−) and (+) epibatidines were of about 1 and 2 μM, respectively (FIG. 5). In contrast, low nanomolar concentrations of (+) or (−) epibatidine were able to activate type II currents (FIG. 4), and the (+) isomer was much more effective than the (−) isomer in eliciting type II currents (FIG. 5). Based on our results, epibatidine would seem to be a more potent and selective agonist for the hippocampal α4β2 nAChR than for the α7-bearing hippocampal nAChR. It remains to be determined whether epibatidine would be even more potent in activating other types of neuronal nAChRs.[39] Epibatidine, in addition to being an extremely potent agonist on neuronal nAChRs, has also been proven to be a highly

specific nicotinic agonist, because it does not interact with other neuronal neurotransmitter receptors, such as GABA receptors, NMDA receptors, serotoninergic receptors, and dopaminergic receptors.[38] Thus, the characterization of the interactions of this alkaloid with CNS nAChRs may be of major importance to the understanding of how these receptors are involved in the analgesia induced by nicotinic agonists.[40]

MODULATION OF nAChR ACTIVITY IN THE MAMMALIAN CNS

Modulation of nAChR Activity by Extracellular Ligands

The coexistence of neuromodulators and neurotransmitters in many synapses is associated with the presence of specific receptor-binding sites that are distinct from the agonist sites and are presumed to recognize the modulator. The NMDA type of glutamatergic receptor, for example, which is involved in learning, memory, and neuronal function, has a number of regulatory sites that are targets for endogenous

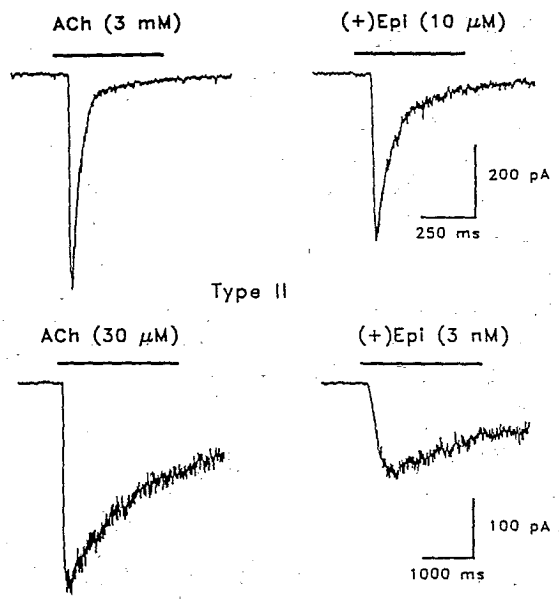

FIGURE 4. Epibatidine-evoked nicotinic whole-cell currents in rat cultured hippocampal neurons. **Top, left:** Sample recording of ACh-activated type IA current in a hippocampal neuron cultured for 21 days. **Top, right:** Sample recording of epibatidine-activated current in the same neuron as in the left panel. **Bottom, left:** Sample recording of ACh-activated type II current in a hippocampal neuron cultured for 11 days. **Bottom, right:** Sample recording of epibatidine-activated current in the same neuron as in the left panel. The nicotinic agonists were applied to the cell every 2 min. Duration of the agonist pulses is indicated by the solid bars on the top of the traces. Holding potential = −56 mV. Currents were classified as type I or II on the basis of the kinetics of their decay phase and their sensitivity to MLA (1 nM) or DHβE (100 nM). Compositions of the external and internal solutions were the same as those described in FIGURE 1.

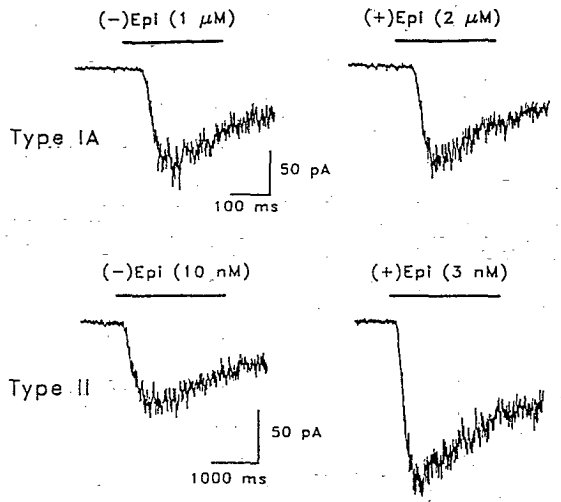

FIGURE 5. Enantiomers of epibatidine distinguish the subtypes of nAChRs present on hippocampal neurons. **Top:** Sample recordings of type IA current activated by (−) (*left*) or (+) (*right*) epibatidine. The recordings were obtained from a hippocampal neuron cultured for 18 days. **Bottom:** Sample recordings of type II current activated by (−) (*left*) or (+) (*right*) epibatidine. The recordings were obtained from a hippocampal neuron cultured for 11 days. The nicotinic agonists were applied to the cell every 2 min. Duration of the agonist pulse is indicated on the top of the traces. Holding potential = −56 mV. Currents were classified as type I or II on the basis of their sensitivity to MLA (1 nM) or DHβE (100 nM). Compositions of the external and internal solutions were the same as those indicated in FIGURE 1.

and exogenous compounds, such as (1) glycine, which is known to decrease NMDA receptor desensitization; (2) Mg^{2+}, MK-801, and phencyclidines, each of which can cause voltage-dependent blockade of NMDA-induced responses via different mechanisms; (3) Zn^{2+}, which allosterically inhibits NMDA receptor activity; and (4) polyamines, which either potentiate or inhibit the activation of NMDA receptors.[41]

In contrast to the vast knowledge on neuromodulators that can bind to the NMDA receptor and control its activation, little is known with respect to neuromodulators that can control nAChR activity in the CNS or at the neuromuscular junction. For example, about 15 years ago, perhydrohistrionicotoxin ($H_{12}HTX$) was shown to have differential effects on end-plate currents (EPCs) depending upon whether the EPCs were activated by nerve stimulation or by iontophoretic application of ACh.[42] It was hypothesized then that an endogenous neuromodulator and ACh could be simultaneously released from the nerve terminal, and that such a modulator could act upon the muscle nAChR to protect it from the inhibitory effect of $H_{12}HTX$ on nerve-evoked EPCs.[42] However, the nature of this putative neuromodulator and its site on action on the nAChR remain obscure to date.

Very recently, a new binding site was identified on neuronal and muscle nAChRs through which the ion-channel activity can be modulated. This site, which is insensitive to ACh, recognizes as agonists the anticholinesterases physostigmine (PHY) and galanthamine (GAL), as well as the muscle relaxant benzoquinonium (BZQ), and the morphine derivative codeine (FIG. 6).[43–51] It was initially shown that

at therapeutically relevant concentrations, PHY can interact directly with the muscle nAChR as an agonist and an open-channel blocker.[43,44] Subsequently, the agonist effect of PHY was observed in several other preparations, each of which expressed different types of nAChRs. Indeed, PHY, as well as BZQ and GAL, can activate the nAChRs expressed on frog muscle fibers, electric organs of *Torpedo,* cultured hippocampal neurons, M10 fibroblasts, and clonal pheochromocytoma (PC12) cells.[43–52] The important conclusion, however, was that PHY, GAL, or BZQ could activate the nAChR channels present in these preparations by binding to a site distinct from that for ACh. Such a conclusion was inferred from the findings that (1) the nAChR-specific monoclonal antibody FK1 can block the agonist actions of these three compounds, without affecting those of ACh, and (2) competitive nicotinic antagonists have no effect on the agonist actions of PHY, GAL, and BZQ, but block those of ACh.[45–53] Supporting the notion that the effects of PHY are mediated via an ACh-insensitive nAChR site, PHY, but not ACh, displaces the binding of FK1 to hippocampal neurons,[48] and PHY can activate ion flux into *Torpedo* vesicles even after the nAChR has been desensitized by high concentrations of ACh.[46]

In an attempt to identify the nAChR binding site for PHY, a study using photoaffinity-labeling techniques was carried out in membrane preparations from *Torpedo* electroplax.[50] In that study, [^{3}H]PHY was activated by ultraviolet light to react covalently with its binding site on the membrane-bound *Torpedo* nAChR, and predominantly the Lys-125 residue of the nAChR α-subunit was found to be radiolabeled. This residue is not situated in the sequence regions presently suggested to contain elements of the ACh-binding site, but is located within the extracellular, amino-terminal region of all the nAChR α subunits cloned to date, a domain suitable for the binding of extracellular ligands[48,54] (FIG. 7). Basically two main domains can be defined in the amino-terminal region of nAChR subunits. One of these domains

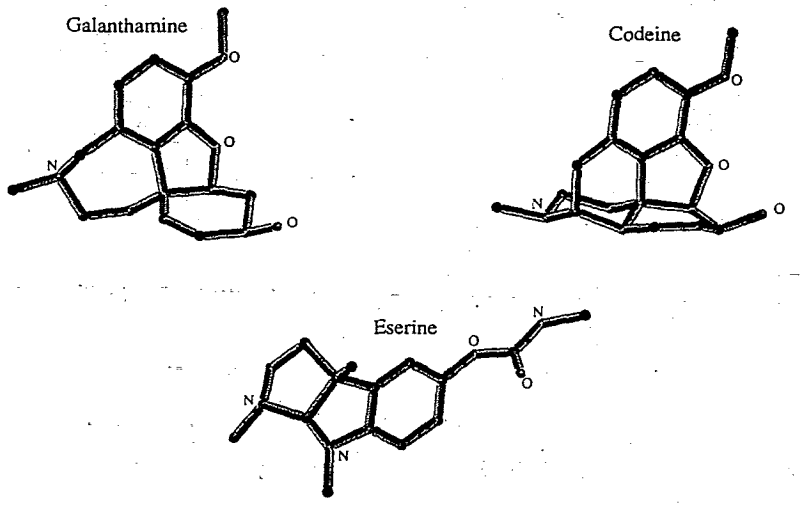

FIGURE 6. Chemical structures of physostigmine, galanthamine, and codeine. The tridimensional structures of physostigmine (eserine), galanthamine, and codeine are illustrated. In the structures, O and N represent the oxygen and nitrogen atoms, respectively. (Adapted from Storch *et al.*[52])

contains the invariant Cys 192 and 193 residues to which ACh and nicotinic antagonists (such as the monoclonal antibody WF6) can bind, and the other includes the invariant Lys-125 to which PHY and related ligands (such as the monoclonal antibody FK1) can bind. Using synthetic peptides that represented the amino acid sequences 181–200 and 118–137 of the *Torpedo* nAChR α subunit, it was demonstrated that whereas FK1 binds with high affinity to the peptide α118–137 and with low affinity to the peptide α181–200, WF6 binds with low affinity to the peptide α118–137 and with high affinity to the peptide α181–200.[54] Taken together with the results from functional and ligand competition studies, these findings indicate that there is practically no overlap between the binding sites for PHY and ACh, and partial overlap between the binding sites for FK1 and WF6. The sequence region of nAChR α subunits in which the binding site for PHY and related compounds is

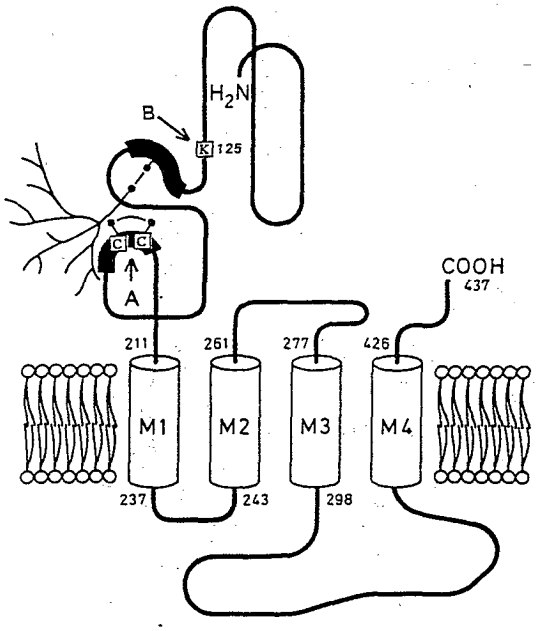

FIGURE 7. Location of the PHY-binding site on the nAChR α subunit. In this model of the nAChR α subunit, the amino acid residues are numbered according to the primary structure of the *Torpedo* nAChR. Arrow A points to the ACh-binding site, and B points to the PHY-binding site (From Pereira *et al.*[48] Reproduced, with permission, from the *Journal of Pharmacology and Experimental Therapeutics.*)

located (α118–137) is amphipathic.[54] The hydrophobicity of the nAChR region that contains the PHY-binding site may account, at least in part, for the ligand selectivity of this site for PHY and related compounds. In fact, in contrast to compounds that bind to the ACh site, which are essentially hydrophilic, all the compounds found to bind to the PHY site are extremely hydrophobic. Inasmuch as the region surrounding and including Lys-125 is highly conserved in all the nAChR α subunits sequenced to date,[48] and PHY has been shown to interact with a variety of nAChR subtypes, it is most likely that the novel identified ligand site on the neuronal nAChR plays a key role in the control of the activation of most, if not all, nAChR subtypes. The identification of an endogenous ligand that could bind to this site and control nAChR activity would ensure the physiological relevance of this newly identified site in the process of nicotinic synaptic transmission.

During the process of synaptic transmission in the neuromuscular junction, depolarization of the presynaptic nerve terminal causes Ca^{2+} influx, mobilization of the ACh-containing vesicles, and increase of ACh release into the synaptic cleft. ACh, then, diffuses across the synaptic cleft from the presynaptic terminal to the muscle, binds to the postsynaptic nAChRs, and increases with high efficacy the probability of opening of the nAChR channel.[55,56] In contrast, the efficacy of PHY and PHY-like compounds as nicotinic agonists, either on muscle or CNS nAChRs, is apparently so low that although these compounds can activate nicotinic single-channel currents they are unable to evoke macroscopic currents.[47,48,51,52] At this stage, one cannot rule out the possibility that these compounds can also exert other effects on the nAChR, which oppose and outweigh their agonist effects, and impair their ability to activate whole-cell responses. Indeed, all the compounds that have been shown to bind to the newly described nAChR site also have open-channel blocking properties or desensitizing effects on the nAChRs, and the concentrations at which such compounds can block or inactivate the nAChR channels overlap those at which they act as agonists.[48,51] Nevertheless, it is tempting to speculate that ligands that would bind to this newly described nAChR site could act as co-agonists rather than agonists, thereby potentiating channel activation by the natural transmitter (unpublished observations). Such a co-agonist action has been observed for glycine on the NMDA receptor.[58,59] Considering that there might be an endogenous ligand that binds to the PHY/GAL site and modulates the CNS nAChR activity, such a ligand could be part of a "chemical network." In such a network, endogenous neurohormones and/or neurotransmitters, in addition to serving their primary receptors, could modulate the activation of the nAChR by ACh.

An attempt has been made to identify endogenous ligands that could bind specifically to the PHY/GAL-binding site.[52] Based on molecular modeling, phenanthrene-type opium alkaloids were found to be structurally related to PHY and GAL (FIG. 8). In outside-out patches excised from PC12 cells, the morphine derivative codeine could activate single-channel currents via the same mechanism as PHY and GAL.[52] Therefore, endogenous opioid-type compounds, for example, endorphin and/or enkephalin, may serve as endogenous ligands for this newly described nAChR-binding site.

Modulation of Activity of CNS nAChRs by Phosphorylation

Receptor phosphorylation is a well-known mechanism by which muscle nAChR activity can be modulated. In the neuromuscular junction, the motoneurons release a peptide named calcitonin-gene-related peptide (CGRP), which can bind to G_s-coupled CGRP receptors located postsynaptically in muscles.[59] Binding of CGRP to its receptors can stimulate cAMP-dependent phosphorylation of muscle nAChRs, which can then be desensitized more rapidly by ACh.[59] The phosphorylation state of proteins closely associated with ion channels has also been associated with the rundown of currents triggered by activation of a number of voltage- and neurotransmitter-gated ion channels. For instance, the rundown of NMDA-activated whole-cell currents and voltage-activated Ca^{2+} currents can be prevented by the use of an ATP-regenerating internal solution.[60,61] The ATP-regenerating internal solution consisted of ATP, phosphocreatine, and creatine phosphokinase, and its main function was to keep the intracellular levels of ATP constant in spite of the dialysis of the intracellular contents by the pipette solution during the whole-cell experiments. Apparently, the mechanism by which ATP prevents the rundown of NMDA-activated currents and voltage-gated Ca^{2+} currents is unrelated to a direct phosphor-

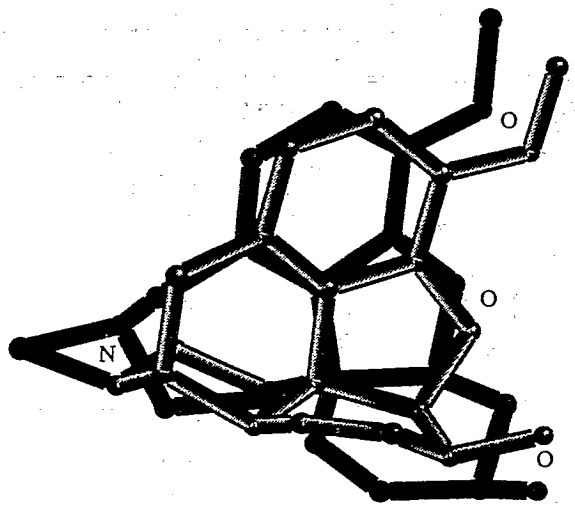

FIGURE 8. Structural relationship between the structures of galanthamine and codeine. The tridimensional chemical structures of galanthamine and codeine are superimposed to depict the common structural features between these two compounds. (Adapted from Storch *et al.*[52])

ylation of the ion-channel protein. Instead, it seems that ATP can influence the activity of these ion channels by altering the state of actin polymerization.[60] A model has been proposed in which Ca^{2+} binds to and modulates the function of a regulatory protein whose interactions with the NMDA receptor are dependent upon the integrity of underlying cytoskeletal elements, particularly actin.[60] This model accounts for both the Ca^{2+} and ATP dependence of rundown of NMDA-activated and voltage-gated Ca^{2+} currents. We have demonstrated that addition of ATP-regenerating compounds to the internal solution can prevent rundown of type IA currents without changing their kinetic properties in cultured hippocampal neurons[17,19] from fetal rats (FIG. 9). Remarkably, phosphocreatine per se is able to prevent the rundown of type IA currents to the same extent as the entire ATP-regenerating solution, in this way suggesting that phosphocreatine may be readily removed during the dialysis of the intracellular contents.[19] It is conceivable that the removal of intracellular phosphocreatine may affect substantially the phosphorylation state of the α7-bearing nAChR, that is, the nAChR that subserves type IA currents, or of proteins closely associated with this nAChR. The phosphorylation/dephosphorylation state of such proteins may not affect the fast nAChR inactivation that accounts for the fast decay of IA currents in the presence of ACh, but may be essential for the slow nAChR inactivation that accounts for the rundown of the currents with time. Under our experimental conditions, the mechanism by which phosphorylation would play a role in preventing rundown of type IA currents seems to be unrelated to the microtubule or microfilament components of the cytoskeleton, because the presence of taxol, a microtubule stabilizer, or phalloidin, a microfilament stabilizer, in the internal solution did not alter the rate of rundown of type IA currents.[19] Of interest, rundown is not observed in type II currents, which are supposedly subserved by an α4β2 neuronal nAChR.

Modulation of Activity of CNS nAChRs by Intracellular Mg^{2+}

Rectification is another property of voltage- and neurotransmitter-gated ion channels that can be modulated. Rectification can occur because of lower probability of channel openings in one range of membrane potentials versus another, or because of a voltage-dependent blockade of the ion channels by a given element. For instance, it is well known that at physiological concentrations extracellular Mg^{2+} can cause a voltage-dependent blockade of NMDA receptors, thereby causing NMDA-activated whole-cell currents to rectify at membrane potentials more negative than −50 mV.[62] Also, ACh-activated currents in PC12 cells show an inward rectification that can be accounted for both by a blockade of the nAChR channels by physiological concentrations of intracellular Mg^{2+} and by a low probability of neuronal nAChR channel opening at positive potentials.[63]

Although most of the recombinant neuronal nAChRs have been known to give rise to currents that rectify inwardly, in our initial studies type IA currents in hippocampal neurons displayed a mild inward rectification at the beginning of the whole-cell current recordings, and this rectification tended to disappear with time.[17,19] These results indicated that a component of the intracellular contents, which could account for the inward rectification of the currents, was probably being removed during the recording by dialysis. Because our internal solution had a high concentra-

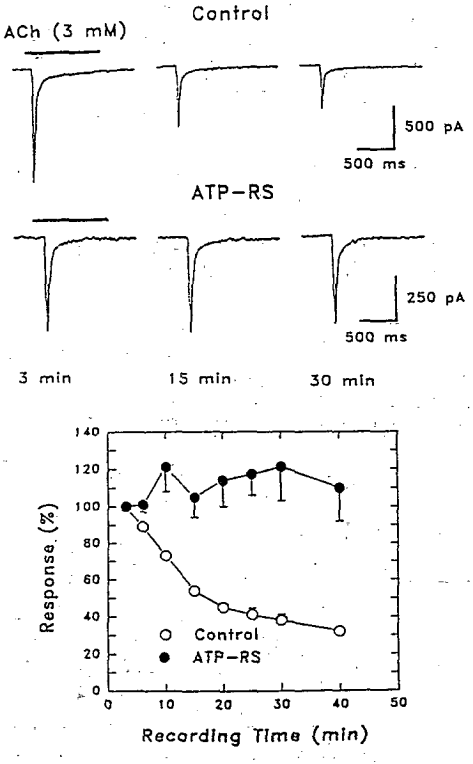

FIGURE 9. ATP-regenerating compounds can prevent the rundown of type IA currents. Top traces represent sample recordings of ACh-evoked whole-cell currents using an internal solution devoid of ATP-regenerating compounds. ACh was applied to a 40-day-old cultured hippocampal neuron in 1-s pulses separated by 2-min intervals. Bottom traces represent sample recordings of ACh-evoked whole-cell currents using an ATP-regenerating internal solution (ATP-RS). ACh was applied to a 20-day-old cultured hippocampal neuron as 1-s pulses separated by 2-min intervals. Holding potential = −56 mV. Bottom graph depicts the effect of the ATP-regenerating solution on the progressive decrease of the peak-current amplitude with time. The peak-current amplitude activated by the first ACh pulse was considered as 100%.

tion of F^-, an anion whose solubility product with Mg^{2+} is extremely low and leads to precipitation of Mg^{2+} in the form of MgF_2, it was hypothesized that intracellular Mg^{2+} could account, at least in part, for the inward rectification of type IA currents, and that its removal from the intracellular medium would result in attenuation of the inward rectification. To test this hypothesis, Mg^{2+}-containing, F^--free internal solutions were used in experiments directed at studying IA current-voltage (I-V) relationships. At the negative range of membrane potentials, the I-V relationship for type IA responses was the same regardless of the presence or absence of Mg^{2+} (2 or 5 mM) in the internal solution. However, at the positive range of membrane potentials, Mg^{2+} in the internal solution inhibited the outward currents. In the range of 0 to 50 mV, the normalized peak-current amplitudes were smaller than those obtained in the absence of added Mg^{2+}.[19] Therefore, under physiological conditions, intracellular Mg^{2+} may play a key role in the control of α-BGT-sensitive nAChR activity in the CNS. In contrast to type IA currents, type II currents rectified both in the presence and in the absence of Mg^{2+} (2 or 5 mM).[19] Thus, whereas intracellular Mg^{2+} may modulate the activity of native α7-bearing CNS nAChRs *in vivo*, it may not be the major factor that affects the activity of native α4β2 CNS nAChRs.

Activation of NMDA receptors in neurons can result in a substantial increase in the intracellular concentrations of free Mg^{2+}.[64] Therefore, although the normal physiological levels of intracellular Mg^{2+} may be enough to prevent to a great extent the activation of α-BGT-sensitive nAChRs at depolarized potentials, it is tempting to speculate that if NMDA receptors and α-BGT-sensitive nAChRs are expressed in the same neuron, when the NMDA receptor is activated at depolarized membrane potentials and the intracellular levels of free Mg^{2+} are increased, the nAChR activation would be completely prevented. Such a cross-talk between the two neurotransmitter systems, that is, the glutamatergic and the cholinergic systems, would be of pivotal relevance to cell function, given that both NMDA receptors and α-BGT-sensitive nAChRs are highly permeable to Ca^{2+}. This cross-talk may represent a means by which rapid rise in intracellular Ca^{2+} concentrations via activation of NMDA receptors and nAChRs could be tightly controlled, so that intracellular Ca^{2+} overloading could be avoided.

ION PERMEABILITY OF α-BGT-SENSITIVE CNS nAChRs

Intracellular Ca^{2+} is important in a variety of physiological processes ranging from modulation of neurotransmitter release to control of cell survival. Several pathways, each of which has distinct temporal characteristics and total capacities, can lead to a rise in intracellular Ca^{2+} concentration.[65] The fastest ones rely upon extracellular Ca^{2+} entry through selective ion channels, such as the voltage-gated Ca^{2+} channels and some neurotransmitter-gated channels. Among the latter, the glutamate-activated NMDA channels are the most widely distributed and best characterized in the CNS. Recent studies have shown that neuronal nAChRs also have a significant Ca^{2+} permeability,[66–71] presenting the interesting possibility that central cholinergic transmission might be involved in fast intracellular Ca^{2+} signals. It is now clear that the Ca^{2+} permeability differs among nAChR subtypes, and that a cellular specificity of the signal may occur depending upon the nAChRs expressed. In the case of chick ciliary ganglion neurons, nAChRs sensitive to α-BGT and MLA induce a marked rise in intracellular Ca^{2+}, whereas α-BGT-insensitive nAChRs in the same cells generate a much smaller Ca^{2+} signal.[71] Also, among the recombinant nAChR channels, the α7-based homomers are unique not only in being sensitive to α-BGT but also in having the highest relative Ca^{2+} permeability.[67–69] Thus, the Ca^{2+}

permeability of the native, α-BGT-sensitive, hippocampal nAChR channel (which probably bears α7-subunits in its structure[17,19]) could be high, and this could be the clue to its physiological function. Recently, our research has been aimed at determining relative ion permeabilities of this nAChR channel in cultured rat hippocampal neurons, according to classical Goldman-Hodgkin-Katz (GHK) modeling.

The reversal potential (V_R) of ACh-induced IA currents was measured using physiological solutions of various compositions.[72–74] In order to ascertain that only the α-BGT-sensitive currents were being tested, it was essential to perform the experiments in the presence of DHβE (0.1 μM) to inhibit the activation of type II (α4β2) nicotinic channels. Permeability ratios relative to Cs^+ were calculated independently of the intracellular medium, using a GHK equation for V_R shifts in the presence of Ca^{2+}. To ascertain the accuracy of the calculations, V_Rs were corrected for liquid-junction potentials, and ion activities were used instead of concentrations. Also, experiments with NMDA-gated currents were carried out in parallel, providing an internal standard for our permeability measurements. Replacing extracellular Cl^- with methanesulfonate caused a negative shift in the ACh current V_R, ruling out a significant contribution of Cl^- to type IA currents. Upon switching from 150 mM Cs^+- to 150 mM Na^+-containing external solutions, the ACh-gated current V_R showed a small positive shift that could be accounted for by the higher Na^+ activity, so that P_{Na}/P_{Cs} was close to the unity. Then, the Ca^{2+} permeability was investigated using Cs^+-based external solutions containing various Ca^{2+} concentrations. The V_R of the ACh-activated currents became more positive when the extracellular Ca^{2+} concentration was raised from 1 to 10 mM (FIG. 10), with the shifts yielding a P_{Ca}/P_{Cs} of about 6. In similar experiments, P_{Ca}/P_{Cs} for the NMDA currents was about 10. Thus, the native α7-bearing nAChR in the rat hippocampus is a cation channel considerably permeable to Ca^{2+}, nearly as much as the NMDA channel.[74] Although both these ACh- and glutamate-gated channels can mediate Ca^{2+} influx, their I-V relationships are quite different, suggesting nonoverlapping roles in the regulation of the neuronal Ca^{2+} concentration.

PHYSIOLOGICAL FUNCTIONS OF CNS nAChRs

The current knowledge of the physiological roles of neuronal nAChRs is still very poor, which is most regrettable in view of the increasing evidence implicating alterations in nAChR function and/or expression in physiopathological processes as in Alzheimer's and Parkinson's diseases.[33,34] Our approach to this problem has been to advance in the characterization of the functional, structural, and pharmacological properties of the brain nAChRs at the molecular and cellular levels.

As alluded to previously, for a number of years the α-BGT-binding proteins in the brain were believed to be unrelated to functional nAChRs.[26] It was not until the cloning of the chick nAChR α7 subunit and the demonstration that this subunit can form a functional ACh-gated ion channel that the α-BGT-binding proteins in the CNS were seen as putative functional nAChRs.[31] Another indirect evidence that CNS α-BGT-binding proteins could be functional nAChRs appeared when affinity-purified α-BGT-binding proteins from the chick optic lobe were shown to yield functional nAChR channels when reconstituted in planar lipid bilayer.[75] The direct demonstration that α-BGT-sensitive currents can be activated in a neuron came with the recordings made in our laboratory,[12–15] soon followed by others.[16,20] This "novel," α-BGT-sensitive receptor channel, which is likely to be distributed in all the brain areas where α-BGT binding and α7-subunit mRNA have been detected, can now be functionally probed. However, the intrinsic properties of this receptor channel,

including fast kinetics and a tendency to rundown, still challenge our technical capabilities. To date, no one has been able to identify the endogenous functional activity of these receptors, like, for instance, an α-BGT-sensitive postsynaptic potential. Although the whole-cell and single-channel currents activated by fast

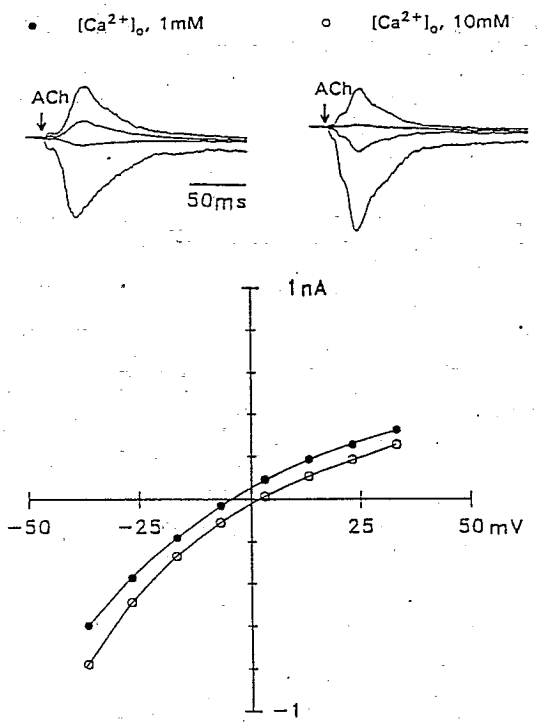

FIGURE 10. Ca^{2+} permeability of α-BGT-sensitive nAChR channels in cultured hippocampal neurons. **Top traces:** Sample recordings of ACh (1 mM)-activated type IA currents in a cultured hippocampal neuron held at the following holding potentials (from top to bottom traces): +23, +3, −7, and −27 mV. The compositions of the external solutions were (in mM): $CsCH_3SO_3$ 100, CsCl 50, HEPES 10, D-glucose 10, $CaCl_2$ 1 (●) or 10 (○), and *N*-methyl-D-glucamine·HCl 35 or 20 (pH, 7.3; osmolarity, 330 mosm). DHβE (0.1 μM), TTX (0.3 μM), and atropine (1 μM) were also added to the external solutions. The composition of the internal solution was (in mM): CsCl 60, CsF 60, CsOH 38.5, $MgCl_2$ 5, EGTA 10, HEPES 10, ATP 5, phosphocreatine 20, Tris·HCl 52.5, and creatine phosphate 50 U/mL (pH, 7.3; osmolarity, 340 mosm). Notice that upon increasing the concentration of extracellular Ca^{2+} from 1 to 10 mM, the currents decayed faster. **Bottom graph:** ACh current-voltage relationship obtained under the two different experimental conditions. Notice that upon increasing the extracellular Ca^{2+} concentration, the reversal potential of ACh current was shifted to the right. ACh (1 mM) was applied to the neuron in a 1-s pulse every 30 s.

application of nicotinic agonists to cultured CNS neurons demonstrate that the α-BGT-sensitive receptors are not presynaptic, it is not clear whether they are located synaptically or extrasynaptically. Electron-microscopic autoradiography studies have shown that α-BGT-binding sites can be located in dendritic and somatic

membranes and that in some cases the labeling is confined to postsynaptic regions.[76] In contrast, in chick ciliary ganglion neurons, α-BGT binding sites occur mostly extrasynaptically, on the somatic membrane.[77] The magnitude and the time course of the changes in agonist concentration in these two circumstances are likely to be quite different, and so must be the dynamics of activation of the α-BGT-sensitive receptor channels.

The data discussed in previous sections showed that α-BGT-sensitive nAChR channels are inward-rectifying cation channels highly permeable to Ca^{2+}. Therefore, they can simultaneously depolarize the neuronal membrane and produce Ca^{2+} influx into the neuronal cells. This influx, in contrast to that mediated by voltage-gated Ca^{2+} channels and NMDA channels, increases with hyperpolarization. The nAChR-mediated rise in intracellular Ca^{2+} concentration can occur even if the current through the channel is not sufficient to depolarize the neuronal membrane. For a given receptor density, the amplitude of that current depends strongly upon the rate of agonist application, because of the fast kinetics of receptor activation/inactivation. Thus, for the nAChRs located in a cholinergic synapse, where the transmitter concentration is supposed to rise and fall sharply, the endogenous response could be an excitatory synaptic potential, associated with an intracellular "Ca^{2+} spike." Alternatively, if the nAChRs are extrasynaptic receptors, which the transmitter presumably reaches in lower concentration after diffusing from a distant source, the response can be a long-lasting Ca^{2+} influx, independent of neuronal excitation. Considering the two extremes, the receptor is capable of modulating both fast and slow Ca^{2+}-dependent processes, ranging from the on-switching of postsynaptic Ca^{2+}-calmodulin-dependent protein kinase II[78] to the activation of immediate early genes.[79] Another process dependent upon Ca^{2+} influx is the assembly of cytoskeletal elements, which may underlie the α-BGT-sensitive nicotinic effects on neurite outgrowth[80] and on retraction of growth cone phylopodia.[81]

The different α-BGT-insensitive nAChRs studied to date are also inward-rectifying cationic channels, but are less permeable to Ca^{2+}, desensitize much more slowly, and have much higher affinities for ACh than the α-BGT-sensitive nAChRs. Several lines of evidence have indicated the presence of these neuronal nAChRs in presynaptic terminals of the peripheral and central nervous systems.[22] A population of nAChRs believed to be composed of a combination of α4 and β2 subunits[82] is located in presynaptic terminals of GABAergic and dopaminergic systems, and can control the release of GABA and dopamine into the synaptic cleft.[21–23] It has been proposed that related nAChRs in the rat interpeduncular nucleus are actually located proximal to the axon terminal in GABAergic neurons, thus being able to trigger fast, tetrodotoxin-sensitive GABA release.[23] In autonomic ganglia, α-BGT-insensitive nAChRs mediate synaptic transmission.[26] However, in the CNS, the physiological role of α-BGT-insensitive postsynaptic nAChRs, which have been detected by whole-cell current recording in neurons from the rat hippocampus (type II and III currents)[17] and habenula,[9] is still unclear.

More studies are required to determine what nAChR subtypes are located at spines on the axodendritic region of CNS neurons, and the role of these receptors in synaptic transmission. It is likely that such nAChRs would be involved in the regulation of synaptic transmission by modulating the release of neurotransmitters from the presynaptic terminals and/or by controlling the activation of second messenger systems at the postsynaptic terminals. Moreover, although acetylcholinesterase is found in high levels throughout the hippocampal region of the brain (FIG. 11), its role in terminating nicotinic responses induced by activation of hippocampal nAChRs remains to be determined.

Another important issue with respect to the physiological roles of CNS nAChRs *in vivo* is related to the existence of a newly described nAChR site through which

nAChR activity may be modulated.[43–54] Although we are still seeking an endogenous neuromodulator that could bind to this site; thereby controlling nAChR activity, the idea of the existence of a neurochemical network that would allow for a single neurotransmitter or neurohormone to act in different receptors by binding to specific recognition sites is exciting. Such a network seems to exist with respect to the

FIGURE 11. Distribution of acetylcholinesterase in a coronal section of the rat dorsal hippocampus. A dense differential labeling of the various hippocampal regions is shown in this micrograph of a cholinesterase-stained hippocampal section. Paraformaldehyde-fixed cryostat sections of rat hippocampi were treated according to a method adapted from Koelle and Fridenwald.[83] Briefly, the sections were air-dried, incubated in a solution containing ethopropazine, glycine, copper sulfate, acetylthiocholine iodide, and sodium acetate, and developed in sodium sulfide·HCl. The labeling was intensified using a silver nitrate solution. In the dendate gyrus, intensely labeled layers (zones) of fibers can be seen in the molecular layer, mainly in the supragranular sublaminae of both the supra- and infralaminar blade of the dendate gyrus, followed by a lighter stained zone and a dense region which extends to the pial surface. Another intensely labeled zone lines the inner border of the granular cell layer towards the hilus of the dendate gyrus, which also displays an intense labeling. In the corpus amonis (CA), differential acetylcholinesterase-staining is also observed. In the CA1 region, the pyramidal cell layer is ensheathed by a broader zone that extends towards the label stratum oriens and a smaller band at the border between the pyramidal layer and the stratum radiatum. The latter and the stratum lacunosum-moleculare—divided by a darker zone—are rather lightly stained. (From Schröder *et al.*, in preparation).

modulation of the NMDA receptor by glycine.[57,58] Glycine, in addition to acting as an inhibitory neurotransmitter by binding to the agonist binding site of glycine-gated Cl^- channels, can also decrease the desensitization of NMDA-induced currents by binding to an allosteric site on the NMDA receptor. Such a mode of action would make centrally acting co-agonists interesting drugs in the treatment of diseases in

which an enhanced sensitivity of a neuroreceptor to its natural transmitter would be advantageous.

In summary, the diversity of CNS nAChRs may represent a means by which differential modulation of receptor activity, desensitization, down- or up-regulation, and cross-talk with other neurotransmitter systems can be mediated by a single neurotransmitter, ACh. It is possible that segregation of different subtypes of CNS nAChRs may occur not only in different areas of the brain, but even more important, in different regions of the neuronal surface. Therefore, in order to unveil the physiological functions of neuronal nAChRs in the CNS, the next step will be to determine the subcellular localization of the diverse nAChR subtypes in various regions of the brain.

SUMMARY

The diversity of neuronal nicotinic receptors (nAChRs) in addition to their possible involvement in such pathological conditions as Alzheimer's disease have directed our research towards the characterization of these receptors in various mammalian brain areas. Our studies have relied on electrophysiological, biochemical, and immunofluorescent techniques applied to cultured and acutely dissociated hippocampal neurons, and have been aimed at identifying the various subtypes of nAChRs expressed in the mammalian central nervous system (CNS), at defining the mechanisms by which CNS nAChR activity is modulated, and at determining the ion permeability of CNS nAChR channels. Our findings can be summarized as follows: (1) hippocampal neurons express at least three subtypes of CNS nAChRs—an α7-subunit-bearing nAChR that subserves fast-inactivating, α-BGT-sensitive currents, which are referred to as type IA, an $\alpha4\beta2$ nAChR that subserves slowly inactivating, dihydro-β-erythroidine-sensitive currents, which are referred to as type II, and an $\alpha3\beta4$ nAChR that subserves slowly inactivating, mecamylamine-sensitive currents, which are referred to as type III; (2) nicotinic agonists can activate a single type of nicotinic current in olfactory bulb neurons, that is, type IA currents; (3) α7-subunit-bearing nAChR channels in the hippocampus have a brief lifetime, a high conductance, and a high Ca^{2+} permeability; (4) the peak amplitude of type IA currents tends to rundown with time, and this rundown can be prevented by the presence of ATP-regenerating compounds (particularly phosphocreatine) in the internal solution; (5) rectification of type IA currents is dependent on the presence of Mg^{2+} in the internal solution; and (6) there is an ACh-insensitive site on neuronal and nonneuronal nAChRs through which the receptor channel can be activated. These findings lay the groundwork for a better understanding of the physiological role of these receptors in synaptic transmission in the CNS.

ACKNOWLEDGMENTS

The technical assistance of M. A. Zelle; B. Marrow, V. Pondeljak, and H. Taschner is gratefully acknowledged. The authors would like to thank Dr. J. G. Montes for his helpful comments on the manuscript. The authors are also grateful to Drs. Ulrich Bolbach and Uwe Metzinger, who participated in the studies of acetylcholinesterase labeling of rat hippocampal sections.

REFERENCES

1. LUKAS, R. J. & E. L. BENNETT. 1980. Interaction of nicotinic receptor affinity reagents with central nervous system α-bungarotoxin binding entities. Mol. Pharmacol. **17:** 149–155.
2. LUKAS, R. J. 1984. Detection of low affinity α-bungarotoxin binding sites in the rat central nervous system. Biochemistry **23:** 1160–1164.
3. CLARKE, P. B. S., R. D. SCHWARTS, S. M. PAUL, C. B. PERT & A. PERT. 1985. Nicotinic binding in rat brain: Autoradiographic comparison of [^{3}H]acetylcholine, [^{3}H]nicotine, and [^{125}I]alpha-bungarotoxin. J. Neurosci. **5:** 1307–1315.
4. SWANSON, L. W., D. M. SIMMONS, P. J. WHITING & J. LINDSTROM. 1987. Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. J. Neurosci. **7:** 3334–3342.
5. SORENSON, E. M. & V. A. CHIAPPINELLI. 1992. Localization of [^{3}H]nicotine, [^{125}I]kappa-bungarotoxin, and [^{125}I]alpha-bungarotoxin binding to nicotinic sites in the chicken forebrain and midbrain. J. Comp. Neurol. **323:** 1–12.
6. ARACAVA, Y., S. S. DESHPANDE, K. L. SWANSON, H. RAPOPORT, S. WONNACOTT, G. LUNT & E. X. ALBUQUERQUE. 1987. Nicotinic acetylcholine receptors in cultured neurons from the hippocampus and brain stem of the rat characterized by single channel recording. FEBS Lett. **222:** 63–70.
7. LIPTON, S. A., E. AIZENMAN & R. H. LORING. 1987. Neuronal nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. Pflügers Arch. **410:** 37–43.
8. ZHANG, Z. W. & P. FELTZ. 1990. Nicotinic acetylcholine receptors in porcine hypophyseal intermediate lobe cells. J. Physiol. (Lond.). **422:** 83–101.
9. MULLE, C. & J.-P. CHANGEUX. 1990. A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp techniques. J. Neurosci. **10:** 169–175.
10. ALKONDON, M. & E. X. ALBUQUERQUE. 1990. α-Cobratoxin blocks the nicotinic acetylcholine receptor in rat hippocampal neurons. Eur. J. Pharmacol. **191:** 505–506.
11. RAMOA, A. S., M. ALKONDON, Y. ARACAVA, J. IRONS, G. G. LUNT, S. S. DESHPANDE, S. WONNACOTT, R. S. ARONSTAM & E. X. ALBUQUERQUE. 1990. The anticonvulsant MK-801 interacts with peripheral and central nicotinic acetylcholine receptor ion channels. J. Pharmacol. Exp. Ther. **254:** 71–82.
12. ALBUQUERQUE, E. X., A. C. S. COSTA, M. ALKONDON, K.-P. SHAW, A. S. RAMOA & Y. ARACAVA. 1991. Functional properties of the nicotinic and glutamatergic receptors. J. Recept. Res. **11:** 603–625.
13. ALKONDON, M. & E. X. ALBUQUERQUE. 1991. Initial characterization of the nicotinic acetylcholine receptors in rat hippocampal neurons. J. Recept. Res. **11:** 1001–1021.
14. ISHIHARA, K., M. ALKONDON, J. G. MONTES & E. X. ALBUQUERQUE. 1995. Nicotinic responses in acutely dissociated rat hippocampal neurons and the selective blockade of fast-desensitizing nicotinic currents by lead. J. Pharmacol. Exp. Ther. In press.
15. ALKONDON, M., E. F. R. PEREIRA, S. WONNACOTT & E. X. ALBUQUERQUE. 1992. Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. Mol. Pharmacol. **41:** 802–808.
16. ZORUMSKI, C. F., L. L. THIO, K. E. ISENBERG & D. B. CLIFFORD. 1992. Nicotinic acetylcholine currents in cultured postnatal rat hippocampal neurons. Mol. Pharmacol. **41:** 931–936.
17. ALKONDON, M. & E. X. ALBUQUERQUE. 1993. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: I. Pharmacological and functional evidence for distinct structural subtypes. J. Pharmacol. Exp. Ther. **265:** 1455–1473.
18. CASTRO, N. G. & E. X. ALBUQUERQUE. 1993. Brief-lifetime, fast-inactivating ion channels account for the α-bungarotoxin-sensitive nicotinic response in hippocampal neurons. Neurosci. Lett. **164:** 137–140.
19. ALKONDON, M., S. REINHARDT, C. LOBRON, B. HERMSEN, A. MAELICKE & E. X. ALBUQUERQUE. 1994. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons: II. Rundown and inward rectification of agonist-elicited whole-cell currents and identification of receptor subunits by *in situ* hybridization. J. Pharmacol. Exp. Ther. **271:** 494–506.
20. ZHANG, Z. W., S. VIJAYARAGHAVAN & D. K. BERG. 1994. Neuronal acetylcholine

receptors that bind α-bungarotoxin with high affinity function as ligand-gated ion channels. Neuron **12:** 167–177.

21. WONNACOTT, S., J. IRONS, G. G. LUNT, C. M. RAPIER & E. X. ALBUQUERQUE. 1988. αBungarotoxin and presynaptic nicotinic receptors: Functional studies. *In* Nicotinic Acetylcholine Receptors in the Nervous System. F. Clementi, C. Gotti & E. Sher, Eds.: 41–60. Springer-Verlag. Berlin.
22. WONNACOTT, S., A. DRASDO, E. SANDERSON & P. POWELL. 1990. Presynaptic nicotinic receptors and the modulation of transmitter release. *In* Ciba Foundation Symposium. The Biology of Nicotine Dependence. 87–105. Wiley. Chichester.
23. LÊNA, C., J.-P. CHANGEUX & C. MULLE. 1993. Evidence for "preterminal" nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus. J. Neurosci. **13:** 2680–2688.
24. CLARKE, P. B. 1993. The fall and rise of neuronal alpha-bungarotoxin binding proteins. Trends Pharmacol. Sci. **13:** 407–413.
25. CHIAPPINELLI, V. A. 1991. Kappa-neurotoxins: Effects on neuronal nicotinic acetylcholine receptors. *In* Snake Toxins. A. L. Harvey, Eds.: 223–258. New York.
26. SARGENT, P. B. 1993. The diversity of neuronal acetylcholine receptors. Annu. Rev. Neurosci. **16:** 403–433.
27. MONTES, J. G., M. ALKONDON, E. F. R. PEREIRA & E. X. ALBUQUERQUE. 1994. Nicotinic acetylcholine receptor of the mammalian central nervous system. *In* Handbook of Membrane Channels: Molecular and Cellular Physiology. C. Peracchia, Ed.: 269–286. Academic Press. San Diego, CA.
28. ELGOYHEN, A. B., D. JOHNSON, J. BOULTER, D. VETTER & S. HEINEMANN. 1994. α9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell **79:** 705–715.
29. DENERIS, E. S., J. CONNOLLY, S. W. ROGERS & R. DUVOISIN. 1991. Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. Trends Pharmacol. Sci. **12:** 34–40.
30. ROLE, L. W. 1992. Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. Curr. Opin. Neurosci. **2:** 254–262.
31. COUTURIER, S., D. BERTRAND, J. M. MATTER, M. C. HERNANDEZ, S. BERTRAND, N. MILLAR, S. VALERA, T. BARKAS & M. BALLIVET. 1990. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homoligomeric channel blocked by αBTX. Neuron **5:** 847–856.
32. GERZANICH, V., R. ANAND & J. LINDSTROM. 1994. Homomers of α8 and α7 subunits of nicotinic receptor exhibit similar channel but contrasting binding site properties. Mol. Pharmacol. **45:** 212–220.
33. SCHRÖDER, H., E. GIACOBINI, R. G. STRUBLE, K. ZILLES & A. MAELICKE. 1991. Nicotinic cholinoceptive neurons of the frontal cortex are reduced in Alzheimer's disease. Neurobiol. Aging **12:** 259–262.
34. LANGE, K. W., F. R. WELLS, P. SENNER & C. D. MARSDEN. 1993. Altered muscarinic and nicotinic receptor densities in cortical and subcortical brain regions in Parkinson's disease. J. Neurochem. **60:** 197–203.
35. MACALLAN, D. R. E., G. G. LUNT, S. WONNACOTT, K. L. SWANSON, H. RAPOPORT & E. X. ALBUQUERQUE. 1988. Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous system. FEBS Lett. **226:** 357–363.
36. ALKONDON, M. & E. X. ALBUQUERQUE. 1994. Presence of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat olfactory bulb neurons. Neurosci. Lett. **176:** 152–156.
37. REVAH, F., D. BERTRAND, J.-L. GALZI, A. DEVILLERIS-THIÉRY, C. MULLE, N. HUSSY, S. BERTRAND, M. BALLIVET & J.-P. CHANGEUX. 1991. Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor. Nature (Lond.) **353:** 846–849.
38. BADIO, B. & J. DALY. 1994. Epibatidine, a potent analgesic and nicotinic agonist. Mol. Pharmacol. **45:** 563–569.
39. ALKONDON, M. & E. X. ALBUQUERQUE. 1994. Enantiomers of epibatidine as potent

nicotinic agonists at two identified subtypes of nicotinic acetylcholine receptors (nAChRs) in rat hippocampal neurons. Soc. Neurosci. Abstr. **20:** 1135.

40. SALLY, T. L. & G. G. BERNSTON. 1979. Antinociceptive effects of central and systemic administration of nicotine in rat. Psychopharmacology **65:** 279–283.
41. SCATTON, B. 1993. The NMDA receptor complex. Fundam. Clin. Pharmacol. **7:** 389–400.
42. ALBUQUERQUE, E. X., P. W. GAGE & A. C. OLIVEIRA. 1979. Differential effect of perhydrohistrionicotoxin on "intrinsic" and "extrinsic" endplate responses. J. Physiol. (Lond.) **297:** 423–442.
43. SHAW, K.-P., Y. ARACAVA, A. AKAIKE, J. W. DALY, D. L. RICKETT & E. X. ALBUQUERQUE. 1985. The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex. Mol. Pharmacol. **28:** 527–538.
44. ALBUQUERQUE, E. X., Y. ARACAVA, W. M. CINTRA, A. BROSSI, B. SCHÖNENBERGER & S. S. DESHPANDE. 1988. Structure-activity relationship of reversible cholinesterase inhibitors: Activation, channel blockade and stereospecificity of the nicotinic acetylcholine receptor-ion channel complex. Braz. J. Med. Biol. Res. **21:** 1173–1196.
45. OKONJO, K. O., J. KUHLMANN & A. MAELICKE. 1991. A second pathway for the activation of the *Torpedo* acetylcholine receptor. Eur. J. Biochem. **200:** 671–677.
46. KUHLMANN, J., K. O. OKONJO & A. MAELICKE. 1991. Desensitization is a property of the cholinergic binding region of the nicotinic acetylcholine receptor, not of the receptor-integral ion channel. FEBS Lett. **279:** 216–218.
47. PEREIRA, E. F. R., M. ALKONDON, T. TANO, N. G. CASTRO, M. M. FRÓES-FERRÃO, R. ROZENTAL, R. S. ARONSTAM & E. X. ALBUQUERQUE. 1993. A novel agonist binding site on nicotinic acetylcholine receptors. J. Recept. Res. **13:** 413–436.
48. PEREIRA, E. F. R., S. REINHARDT-MAELICKE, A. SCHRATTENHOLZ, A. MAELICKE & E. X. ALBUQUERQUE. 1993. Identification and characterization of a new agonist site on nicotinic acetylcholine receptors of cultured hippocampal neurons. J. Pharmacol. Exp. Ther. **265:** 1474–1491.
49. SCHRATTENHOLZ, A., T. COBAN, B. SCHRÖDER, K. O. OKONJO, J. KUHLMANN, E. F. R. PEREIRA, E. X. ALBUQUERQUE & A. MAELICKE. 1993. Biochemical characterization of a novel channel-activating site on nicotinic acetylcholine receptors. J. Recept. Res. **13:** 393–412.
50. SCHRATTENHOLZ, A., J. GODOVAC-ZIMMERMAN, H.-J. SCHÄFER, E. X. ALBUQUERQUE & A. MAELICKE. 1993. Photoaffinity labeling of *Torpedo* acetylcholine receptor by the reversible cholinesterase inhibitor physostigmine. Eur. J. Biochem. **216:** 671–677.
51. PEREIRA, E. F. R., M. ALKONDON, S. REINHARDT, A. MAELICKE, X. PENG, J. LINDSTROM, P. WHITING & E. X. ALBUQUERQUE. 1994. Physostigmine and galanthamine characterize the presence of the novel binding site on the $\alpha 4\beta 2$ subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells. J. Pharmacol. Exp. Ther. **270:** 768–778.
52. STORCH, A., J. C. COOPER, O. GUTBROD, K.-H. WEBER, S. REINHARDT, C. LOBRON, B. HERMSEN, V. SOSKIC, A. SCHRATTENHOLZ, E. X. ALBUQUERQUE, C. METHFESSEL & A. MAELICKE. 1994. Physostigmine and galanthamine act as non-competitive agonists on clonal rat pheochromocytoma cells. Eur. J. Pharmacol. Submitted.
53. DUNN, S. M. J. & M. A. RAFTERY. 1993. Cholinergic binding sites on the pentameric acetylcholine receptor of *Torpedo californica*. Biochemistry **32:** 8608–8615.
54. SCHRÖDER, B., S. REINHARDT-MAELICKE, A. SCHRATTENHOLZ, K. E. MCLANE, B. M. CONTI-TRONCONI & A. MAELICKE. 1994. Monoclonal antibodies FK1 and WF6 define two neighboring ligand binding sites on *Torpedo* acetylcholine receptor α-polypeptide. J. Biol. Chem. **269:** 10407–10416.
55. TRAUTMANN, A. 1983. A comparative study of the activation of the cholinergic receptor by various agonists. Proc. R. Soc. Lond. Biol. Sci. **218:** 241–251.
56. KARLIN, A. 1993. Structure of nicotinic acetylcholine receptors. Curr. Opin. Neurobiol. **3:** 299–309.
57. BENVENISTE, M., J. CLEMENTES, L. VYCKLICKY & M. L. MAYER. 1990. A kinetic analysis of the modulation of *N*-methyl-D-aspartate receptors by glycine in mouse cultured hippocampal neurons. J. Physiol. (Lond.) **428:** 337–357.

58. JOHNSON, J. & P. ASCHER. 1992. Equilibrium and kinetic study of glycine action on the NMDA receptor in cultured mouse brain neurons. J. Physiol. (Lond.) **455:** 339–365.
59. MILES, K., P. GREENGARD & R. L. HUGANIR. 1989. Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. Neuron **2:** 1517–1524.
60. ROSEMUND, C. & G. L. WESTBROOK. 1993. Calcium-induced actin depolymerization reduces NMDA channel activity. Neuron **10:** 805–814.
61. JOHNSON, B. D. & L. BYERLY. 1993. A cytoskeletal mechanism for calcium channel metabolic dependence and inactivation by intracellular calcium. Neuron **10:** 797–804.
62. NELSON, M. & E. X. ALBUQUERQUE. 1994. 9-Aminoacridines act at a site different from that for Mg^{2+} in blockade of the *N*-methyl-D-aspartate receptor channel. Mol. Pharmacol. **46:** 151–160.
63. IFUNE, C. K. & J. H. STEINBACH. 1992. Inward rectification of acetylcholine-elicited currents in rat pheochromocytoma cells. J. Physiol. (Lond.) **457:** 143–165.
64. BROCARD, J. B., S. RAJDEV & I. J. REYNOLDS. 1993. Glutamate-induced increases in intracellular free Mg^{2+} in cultured cortical neurons. Neuron **11:** 751–757.
65. BLAUSTEIN, M. P. 1988. Calcium transport and buffering in neurons. Trends Neurosci. **11:** 438–443.
66. MULLE, C., D. COQUET, H. KORN & J.-P. CHANGEUX. 1992. Calcium influx through nicotinic receptors in rat central neurons: Its relevance to cellular regulation. Neuron **8:** 135–143.
67. SÉGUÉLA, P., J. WADICHE, K. DINELY-MILLER, J. A. DANI & J. W. PATRICK. 1993. Molecular cloning, functional properties, and distribution of rat brain α7: A nicotinic cation channel highly permeable to Ca^{2+}. J. Neurosci. **13:** 596–604.
68. SANDS. S. B., A. C. S. COSTA & J. W. PATRICK. 1993. Barium permeability of neuronal nicotinic receptor α7 expressed in *Xenopus* oocytes. Biophys. J. **65:** 2614–2621.
69. BERTRAND, D., N. L. GALZI, A. DEVILLERS-THIERY, S. BERTRAND & J.-P. CHANGEUX. 1993. Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal α7 nicotinic receptor. 1993. Proc. Natl. Acad. Sci. USA **90:** 6971–6975.
70. VERNINO, S., M. AMADOR, C. W. LUETJE, J. PATRICK & J. A. DANI. 1992. Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron **8:** 127–134.
71. VIJAYARAGHAVAN, S., P. C. PUGH, Z.-W. ZANG, M. M. RAFTERY & D. K. BERG. 1992. Nicotinic receptors that bind α-bungarotoxin on neurons raise intracellular free calcium. Neuron **8:** 353–362.
72. CASTRO, N. G., A. T. ELDEFRAWI & E. X. ALBUQUERQUE. 1993. Fast kinetics and calcium permeability of α-bungarotoxin-sensitive hippocampal nicotinic receptor channels. Soc. Neurosci. Abstr. **19:** 464.
73. CASTRO, N. G. & E. X. ALBUQUERQUE. 1994. Calcium permeability of α-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal neurons. Biophys. J. **66:** A214.
74. CASTRO, N. G. & E. X. ALBUQUERQUE. 1995. The α-bungarotoxin-sensitive hippocampal nicotinic acetylcholine receptor channel has a high calcium permeability. Biophys. J. In press.
75. GOTTI, C., A. ESPARIS OGANDO, W. HANKE, R. SCHLUE. M. MORETTI & F. CLEMENTI. 1991. Purification and characterization of an α-bungarotoxin receptor that forms a functional nicotinic channel. Proc. Natl. Acad. Sci. USA **88:** 3258–3262.
76. HUNT, S. P. & J. SCHMIDT. 1978. The electron microscopy autoradiographic localization of α-bungarotoxin binding sites within the central nervous system of the rat. Brain Res. **142:** 152–159.
77. JACOB, M. H. & D. K. BERG. 1983. The ultrastructural localization of alpha-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J. Neurosci. **3:** 260–271.
78. BRONSTEIN, J. M., D. B. FARBER & C. G. WASTERLAIN. 1993. Regulation of type-II calmodulin kinase: Functional implications. Brain Res. Rev. **18:** 135–147.

79. BADING, H., D. D. GINTY & M. E. GREENBERG. 1993. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science **260:** 181–186.
80. PUGH, P. C. & D. K. BERG. 1994. Neuronal acetylcholine receptors that bind α-bungarotoxin mediate neurite retraction in a calcium-dependent manner. J. Neurosci. **14:** 889–896.
81. CHAN, J. & M. QUIK. 1993. A role for the nicotinic α-bungarotoxin receptor in neurite outgrowth in PC12 cells. Neuroscience **56:** 441–451.
82. WHITING, P., R. SCHOEPFER, J. LINDSTROM & T. PRIESTLEY. 1991. Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably transfected in mouse fibroblasts. Mol. Pharmacol. **40:** 463–472.
83. KOELLE, G. B. & J. S. FRIEDENWALD. 1949. A histochemical method for localizing cholinesterase activity. Proc. Soc. Exp. Biol. Med. **70:** 617–622.